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Production of high quality, healthy ornamental crops through meristem culture

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SUMMARY

An increasing number of ornamental crops are being multiplied *in vitro*. In this article, the application of meristem culture to plants free from viruses is reviewed and is shown to be useful and cost-effective. First, a literature survey is given on results of meristem culture applied to ornamentals and on damage due to virus diseases. Secondly trials and results with lily, *Alstroemeria* and *Delphinium* and their viruses are discussed.

Virus-free plants have a considerable surplus value compared with infected plants. Additional advantages are that a virus-free crop cannot be a source of contamination for other varieties of the same crop, and that there are no phytosanitary limitations. Virus-free does not mean resistant, however, and re-infection is possible, but can be prevented by strict measures.

Meristem culture applied to *in vitro* cultures of lily, *Alstroemeria* and *Delphinium* always yielded a much lower amount of virus-free plantlets than the usual procedure, i.e. meristem culture applied to plants grown in the glasshouse.

Key-words: Alstroemeria, Delphinium, ELISA, lily, meristem culture, tissue culture, virus-free.

INTRODUCTION

Forty years ago, meristem culture was applied for the first time to free virus-infected dahlia plants from virus (Morel & Martin 1952). The healthy shoots grown from the meristems had to be grafted on young, virus-free seedlings as roots were not yet obtained. Tissue-culture techniques have since been improved and dahlia meristems later yielded rooted shoots (Mullin & Schlegel 1978). Meristem culture has now been applied to many crops, especially with the intent of eliminating certain viruses. The subject has been reviewed by Hollings (1965), Mori *et al.* (1969), Quak (1977), Wang & Hu (1980), Quak (1982) and Kartha (1986). These review articles deal with various topics: dimensions of the meristem, degree of virus elimination (some viruses are more readily eliminated than others), speculations about possible causes of virus elimination by meristem culture, the dependence on reliable and sensitive detection methods and the permanent danger of re-infection.

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During the last 10 years, there were only a few new cases reported of successful trials to free certain ornamentals from specific viruses. However, much information has been gathered on those viruses and the detection methods have been improved and simplified. Large-scale testing for commercial purposes is more feasible than before. To the list of ornamentals that can be made virus-free, have been added: *Aeschynanthus hildebrandii*, free from the difficult to eliminate tobacco mosaic virus (Paludan 1985b); *Alstroemeria*, free from both *Alstroemeria* mosaic virus and *Alstroemeria* carla virus (Hakkaart & Versluijs 1985, 1988); *Cymbidium* sp., free not only from *Cymbidium* mosaic virus which had been reported earlier by others, but also from *Odontoglossum* ringspot virus, a strain of tobacco mosaic virus (Inouye 1984); *Euphorbia pulcherrima*, free from both poinsettia mosaic virus and poinsettia cryptic virus (Paludan & Begtrup 1986); freesia, 25 varieties, from freesia mosaic virus, bean yellow mosaic virus and cucumber mosaic virus (Bertaccini *et al.* 1989); *Kalanchoe* from *Kalanchoe* latent virus (Paludan 1985c); and *Laeliocattleya areca*, from *Cymbidium* mosaic virus but not from *Odontoglossum* ringspot virus (Ishii 1974). Kromer & Kukulczanka (1985) described successful trials to apply meristem culture to *Canna indica*, but did not test for the presence of viruses.

Meristem culture may yield virus-free plants, but also plants free from bacteria and fungi (Pierik 1987), viroids (Paludan 1985a) and mycoplasms (Green *et al.* 1989). Viroids are smaller than viruses, are composed of nucleic acid without the protein coat of viruses and can be extremely harmful. Chrysanthemum stunt viroid can be eliminated by a cold treatment of plants at 5°C for 6 months, followed by meristem culture. Paduch-Cichal & Kryczynski (1987) applied this combined treatment to chrysanthemums and potatoes and obtained 18·5–80% viroid-free plants, dependent on the type of viroid and on the kind of plant material. Ulrychova & Petru (1975) had already predicted, after their research work on mycoplasma-contaminated callus cultures of *Nicotiana glauca*, that plants can be freed from mycoplasms.

Nematodes, particularly those that live inside the roots like *Pratylenchus* species, most probably can also be eliminated through meristem culture, but there are no references on this subject.

The application of meristem culture to obtain virus-free plants will be the main topic of this article, but some attention will be given to meristem culture and bacteria.

It should be noted that the term 'virus-free plants' is not correct: the plants are free from the viruses for which has been tested. The term 'virus-tested' would be more accurate (F. Quak, pers. comm.).

MERISTEM CULTURE AND VIRUSES

Virus damage

The damage caused by various viruses, alone or together, can be tremendous. Crop loss in the USA due to plant virus infections has been estimated at $1·5–2·0 billion annually (Bialy & Klausner 1986). In 1982 Bos reviewed crop losses due to viruses and discussed the difficulties encountered in damage evaluation. Barnett (1988) confined his review article to virus damage in ornamental crops. He distinguished between direct damage categories such as reduction in growth, reduction in vigour, and reduction in quality or market value and indirect damage related to costs of maintaining crop health. In ornamental crops, uniform, high quality plants must be produced; virus diseases can affect visual attractiveness and can lead to irregularity in production schedules.

Hakkaart (1964) inoculated plants of a virus-free carnation variety with four carnation viruses. Three of those reduced the number of flowers per plant and decreased the quality of the flowers. A combination of two viruses resulted in more severe symptoms than those produced by each virus alone. He found that even a virus, which does not yield visible symptoms, like mottle, may adversely affect the yield.

Gippert & Schmelzer (1973) eliminated viruses from a number of *Pelargonium zonale* varieties through meristem culture. Although viruses do not always cause symptoms in *Pelargonium*, the plants grown from meristems were more vigorous than untreated plants and produced 20–30% more cuttings. In addition, rooting of the cuttings was improved, so that the total production increased by about 36%. In commercial application these results have been confirmed.

Broken tulip flowers were once sought because of their beauty (Schenk 1976). Now it is known that tulip breaking virus (TBV), the cause of this phenomenon, is undesirable as it leads to degeneration of the bulb stocks. TBV may also cause severe symptoms in lily, particularly if it occurs together with lily symptomless virus, LSV (Asjes *et al.* 1973; Derks & Abbink 1988). The bulbs of severely infected lilies are smaller than those of healthy plants and diseased bulbs produce shorter plants. Infected plants show various symptoms and die too early. The vase life is shorter than that of virus-free lilies (Asjes *et al.* 1973). Boontjes (1983) demonstrated that leaves of virus-free lilies remained green for a much longer period than those of virus-infected lilies; virus-free lilies showed a 20–40% better growth than infected ones (measured by bulb production). According to Derks & Hendriks (1990), lily symptomless virus was involved in the trials of Boontjes, but it clearly has been named incorrectly. The more recently discovered lily virus X (LVX) is less harmful than LSV but causes, in combination with LSV, an aggravation of LSV-symptoms (Derks & Hendriks 1990).

Virus-free irises gave a *c.* 50% higher yield (i.e. bulbs) compared with virus-infected plants. Rijnders (1989) calculated that, although it will take about 4 years before the profits exceed the costs, the investments will be, in the long run, amply returned.

Meristem culture of lily

Some years ago the Bulb Inspection Service and the Dutch lily growers started a unique project to achieve a stepwise decrease in the, already low, presence of lily viruses down to a figure close to zero in 1994 (Anonymous 1989). Tissue culture companies can play an important role in the project, as the application of meristem culture can keep lilies free from LSV (Asjes *et al.* 1974; Van Aartrijk & Blom-Barnhoorn, 1978), and TBV (Blom-Barnhoorn & Van Aartrijk, 1985). LVX does not frequently occur, so usually LVX-free starting material can be selected. It is possible to detect all three viruses, even if present at low concentrations, through Enzyme Linked Immunosorbent Assay (ELISA) as good antisera are available (Derks & Vink-van den Abeele 1980; Derks *et al.* 1982, 1986). As a rule, the starting material is indexed first by testing at least two scales per bulb (Van Schadewijk 1986). As soon as the *in vitro* bulblets grown from the meristems are adequately developed, the *in vitro* material is tested for the virus (or the viruses) present in the starting material. Later the *in vitro* bulblets are tested a second time. The remaining bulblets are cold-treated and planted in a glasshouse. Leaves of these bulblets are used for a third and final examination (Van Aartrijk & Blom-Barnhoorn 1978; Blom-Barnhoorn & Van Aartrijk 1985). Testing of virus in the *in vitro* material is not quite reliable for LSV and not at all reliable for TBV, thus the third indexing of leaf material grown in the glasshouse is absolutely necessary. Prior to virus testing, the starting material should be stored for 3

Table 1. Percentages of virus-free lily cultures, obtained after application of meristem culture to *in vitro* cultures and to bulb scales lifted from the soil

	Meristems from:				
	In vitro scales		Bulbs from the soil		
Variety	Virus	% virus-free	Variety	Virus	% virus-free
A	LSV	25	D	LSV	75
B	LSV	4	E	LSV	77
C	LSV+TBV	6	F	LSV	98
			G	LSV	67

weeks at about 2°C to yield reliable results (Van Schadewijk 1986). *Longiflorum* spp., however, require storage at 20°C for detection of TBV (Asjes *et al.* 1989).

In our tissue-culture laboratory *c.* 150 different varieties have been brought in during a period of more than 4 years. Only 10 were completely virus-infected (usually with LSV). These 10 varieties have successfully been meristemmed and tested according to the above-mentioned procedures.

In spite of a thorough surface-sterilization, lily scales often yield many fungal and bacterial contaminations. Therefore, we tried to obtain meristems from *in vitro* cultures. The resulting percentage of virus-free bulblets, however, appeared to be substantially lower than with the current procedure (Table 1).

Meristem culture of Alstroemeria

Alstroemeria has been multiplied vegetatively for many years, so viruses occurring in the crop have also been multiplied for many years. The genus *Alstroemeria* comprises a number of groups, e.g. the Butterfly-group, the Aurea-group and the Orchid-group. Butterflies usually are difficult in tissue culture, in contrast with, for instance, Orchids. *Alstroemeria's* of the Butterfly-type nearly always contain *Alstroemeria's* mosaic virus (AlMV; Brunt & Phillips 1981; Hakkaart & Versluijs 1985). The Aurea-type *Alstroemeria's* usually do not contain AlMV, but they occasionally do contain *Alstroemeria* carla virus (AlCV) and cucumber mosaic virus (CMV). AlCV and CMV sometimes occur in the other *Alstroemeria* types. AlCV is closely related to LSV (Phillips & Brunt 1986). CMV was first reported in *Alstroemeria* by Hakkaart (1986). All three viruses can be detected through ELISA as good antisera are available (Maat 1980, 1983). *Alstroemeria* may also contain some uncharacterized viruses, for instance a virus that causes flower colour breaking.

Symptoms induced by AlMV, the most frequently occurring virus, are streaking of the leaf which shows like light green and dark spots parallel to the midrib. Sometimes a few dark stripes are visible on the flower petals.

Several varieties have been freed from AlMV and AlCV by meristem culture (Hakkaart & Versluijs 1985, 1988). In our laboratory all commercial varieties, mainly Butterflies, have now been made virus-free. In the case of *Alstroemeria*, virus tests of the *in vitro* material grown from meristems gave satisfactory results, as with the potato viruses X and S (Gallenberg & Jones 1985). The *in vitro Alstroemeria* plantlets were tested twice for the virus(es) present in the starting material, with an interval of at least 1 month (preferably 2

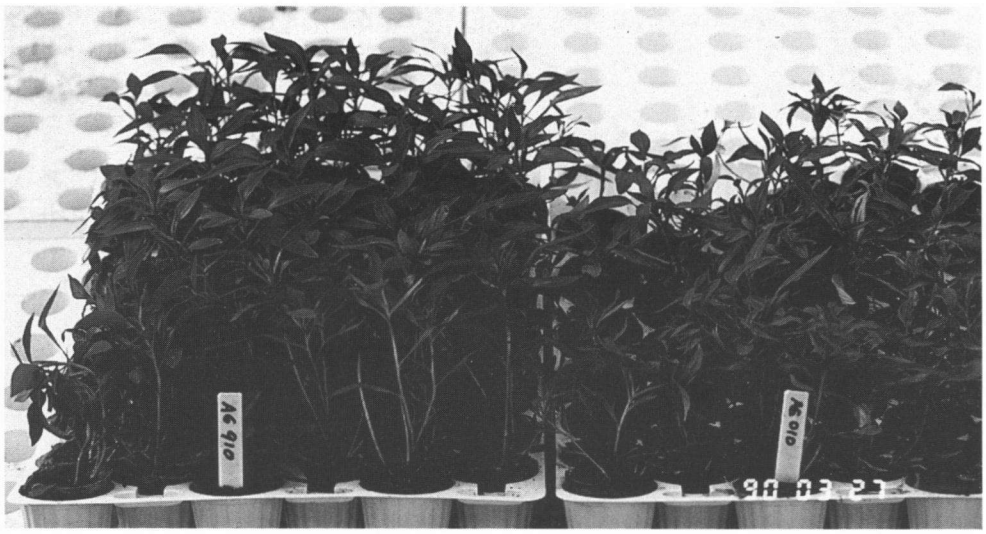

Fig. 1. *Alstroemeria* plants of variety Snow Queen, just before planting in a glasshouse for comparison of yield and quality, of virus-infected plants (right) and virus-free plants from meristems (left).

Table 2. Interim results, after 10 weeks of harvesting, of a comparison between 20 virus-free plants from meristems and 20 plants infected with *Alstroemeria* mosaic virus and *Alstroemeria* carla virus of the variety Snow Queen, grown in a glasshouse

	Infected	Virus-free
Number of flower stems per plant (on average)	19·2	28·4
1st quality	65%	76%
2nd quality	35%	24%
Leaf senescence	Very fast	Not noticeable
Vase life (number of days)		
Flower	22	22
Leaf	21	25
Yellowing of leaf	After 10 days	Not noticeable

or more). A test after weaning of the plantlets in the glasshouse, as required for lily, is not necessary as it does not yield additional information.

It is assumed that virus-infected plants yield minor quality flowers, but data were missing as were data on a possible detrimental effect on the yield. Therefore, 20 virus-free plants from meristems of the variety Snow Queen, a Butterfly-type, were planted in a glasshouse next to 20 plants of the same variety containing AlMV and AlCV (Fig. 1).

Although this design is not optimal as the virus-free plants will get re-infected sooner or later, it was chosen to reveal the differences between virus-free and infected plants under the same conditions. After a harvesting period of 10 weeks, the interim results were collected (Table 2). The results show that the virus-free 'Snow Queen' had in this trial, in

Table 3. Numbers and percentage of virus-free *Alstroemeria* plantlets, obtained after application of meristem culture to *in vitro* cultures and to mother plants

	Meristems from:	
	In vitro plantlets	Plants from the glasshouse
Variety		
Snowflake	0 (7)*	28 (31)
Yvonne van Rooij	3 (6)	12 (12)
Melody	2 (3)	20 (23)
86-264	1 (12)	21 (23)
Rumba	2 (4)	16 (19)
Total	8 (32) = 25%	97 (108) = 90%

*In brackets: total number of initiated and developed meristems.

comparison with infected plants, a 50% increase in yield, more first quality flower stems, better quality of the leaves, and a longer vase life as the leaves did not yellow.

The 50% increase in yield may be an exception and may have resulted from the presence of two viruses in the infected plants. Ten months after the start of this trial in the glasshouse, the virus-free plants were re-infected, most probably through the knife used for cutting the flower stems, as specific measures to prevent re-infection had not been taken. In the weeks after these results had been registered, the trial had a similar progress as to the difference between healthy and infected, in spite of the recent re-infection.

From the results it was calculated, that the difference in output between a virus-infected and a virus-free plant of 'Snow Queen' amounted in this case to *c.* Dfl.10 per year. The extra labour required for the application of meristem culture is estimated, per cultivar, to be 52 h. This figure is based on initiation of 80 meristems in 2 days; included is the labour for the extra transfers until the virus-indexing tests can be done, and also the labour for these tests. It is easy to calculate when the application of meristem culture will be rewarding.

The meristems are located in the rhizomes in the soil and often give rise to contaminated *in vitro* cultures. To avoid contaminations, meristem culture was applied to *in vitro* plantlets. The percentage of virus-free plantlets of five varieties, however, was much lower than when meristems were isolated from the unsterile mother plants in the glasshouse (Table 3). A possible explanation for the low efficiency of meristem culture from *in vitro* lily and *Alstroemeria* plantlets is an increase of the virus multiplication at the constant temperature of 23°C, the *in vitro* growth temperature of both crops.

Meristem culture of Delphinium

In another crop that is multiplied vegetatively, *Delphinium*, CMV was found occasionally in our laboratory. CMV in *Delphinium* could be detected with the same antiserum used for detection of CMV in *Alstroemeria* (Maat 1980). Indexing of the mother material, however, was not simple as the distribution of the virus in the various plant parts seemed to be erratic. CMV in *Delphinium* is not regarded as a serious problem (Edwards 1989). However, CMV-infected plants derived from tissue culture and planted in the field either did not survive or remained stunted and dwarfed. In these plants and in tissue-cultured,

CMV-infected plants grown in the glasshouse, the flower colour faded, flower breaking occurred, flowers or even flower stems could be deformed, leaves sometimes showed a mosaic pattern and could be narrowed with a distinct pointed appearance. Such infected plants are a source of contamination to healthy plants in the same plot or glasshouse, as the virus can easily be transmitted with the sap of diseased plants (for instance through a knife) and by aphids. CMV can also be transmitted through the seed to the progeny.

Meristem culture of *Delphinium* was for the first time applied in our laboratory to *in vitro* cultures infected with CMV. The plantlets grown from the meristems were tested twice for CMV *in vitro*. A third test of weaned plantlets growing in the glasshouse still detected some virus-infected plants and thus appeared to be necessary. Eventually a small number of virus-free clones was obtained: only seven out of a total number of 88 (about 8%). As with lily and *Alstroemeria*, it was troublesome to obtain virus-free *Delphinium* plants by application of meristem culture to *in vitro* material.

MERISTEM CULTURE AND BACTERIA

Only few publications deal with meristem culture to free plants from bacteria (Pierik 1987). Meristem culture, however, is not the solution for the great number of problems due to bacterial contamination in tissue culture. Bastiaens (1983), Leiffert *et al.* (1989) and Leiffert & Waites (1990) reported on bacterial contaminations in tissue culture and stressed that not only plant pathogenic but also saprophytic organisms should be avoided. Some years ago a symposium on (bacterial) contaminations was held (Cassells 1988).

Bastiaens (1983) already mentioned the occurrence of 'endogenous' bacteria in plant tissues. Nowadays it is known that many plant tissues contain so-called endophytes (e.g. Misaghi & Donndelinger 1990). It is not yet clear, whether plants should always be freed from these organisms. A variety of statice (*Limonium sinuatum*) multiplied in tissue culture in our laboratory contained a *Flavobacterium* sp., which stimulated the growth and especially the rooting of *in vitro* cultures. This was clearly demonstrated in a number of trials in the glasshouse in which plantlets with the bacteria were compared with plantlets free from the bacteria.

CONCLUSION

Meristem culture is an extremely useful technique, for it results in high quality, healthy crops with increased yields which are no longer a source of contamination. A further advantage is that there are no phytosanitary limitations for such crops. The disease-free crops can be stored *in vitro* to avoid re-infection, and then multiplied whenever wanted. The effect of meristem culture may, in critical cases, be improved by a heat treatment (Nyland & Goheen 1969) or by chemotherapeutica (Long & Cassells 1986). Virazole (active ingredient: ribavirine; Lerch 1987) yielded positive results in lily for LSV alone or LSV plus TBV, but not for TBV alone (Blom-Barnhoorn & Van Aartrijk 1985).

As a part of the process of large-scale fast multiplication of crops through tissue culture, a more and more applied technique, meristem culture is a 'must' in order to eliminate certain viruses. Of equal importance are reliable tests for viruses, preceded by making an inventory and identification of those viruses.

A problem that cannot be solved by application of meristem culture is the occurrence of re-infection. Certain measures, if well implemented, can prevent re-infection. The measures differ per virus and depend on the way of spread. Resistance, however, is the

ultimate solution to the problem of re-infection, but it will take a long time before crops will be made resistant to the most harmful organisms and viruses.

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